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The options for the treatment of dermatophytosis being limited, an effective and safe antifungal therapy is highly desired. Fungi-Free™ has the potential for use in the treatment of dermatophytosis. Guinea pigs are susceptible to dermatophytosis similar to humans. Their large body surface provides sufficient area to perform experiments. Thus, a guinea pig model of dermatophytosis has been used in efficacy studies of antifungal agents.

An inoculum of 1×10^7 *T. mentagrophytes* cells was applied on abraded skin of anesthetized guinea pigs to establish dermatophytosis. Efficacy of Fungi-Free™ cream at two different doses was evaluated. Clinical scoring was done based on visual examination of the infected site, and mycological evaluation was performed using hair root invasion test for fungal growth. Histopathologic analysis was also performed. Clinical and mycological evaluation showed that once or twice daily administration of Fungi-Free™ was highly effective in the treatment of guinea pig dermatophytosis.

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Study Title:

Determination of the Efficacy of Fungi-Free™ as a Topical Treatment of Dermatophytosis using a Guinea Pig Model

Introduction

Dermatophytes can cause a variety of superficial mycoses such as, ringworm, athlete's foot and jock itch that are recognized as a significant health concern. Despite increase in the awareness and development of new antifungal agents, complete cure is often not possible with intractability, re-infection and/or relapse occurring in about 15% of patients with dermatophytosis. Therefore, search for more efficacious and safe anti-dermatophytic agents continues (1, 2).

Fungi-Free™ cream, developed by Ganeden Biotech, is commercially available in the USA as an over-the-counter medication for the treatment of dermatophytosis. Fungi-Free™ is composed of a bacterial supernatant (containing a complex fermentation product) associated with miconazole (an FDA-approved drug). The other ingredients in Fungi-Free™ are chemical substances that are safe and commonly used in topical preparations. The antifungal effect of Fungi-Free™ is due to the bacterial supernatant in addition to miconazole. The bacterial supernatant has been tested *in vitro* for its antifungal activity. In our preliminary *in vitro* study, the supernatant was found to be highly effective, capable of $\geq 80\%$ inhibition against *T. rubrum* and *T. mentagrophytes*, and active against *C. albicans* and *C. parapsilosis* among several pathogenic fungi. No adverse reactions have been reported during two-year period of clinical use

of Fungi-Free™. A preliminary clinical study using a repeat insult patch test in 50 participants showed that Fungi-Free™ was a safe medication as no adverse reaction was reported [Data on file at Ganeden]. Additionally, preliminary data from a study conducted by Dr. Joan Meyer showed that 89% of the 23 patients enrolled in a clinical trial to evaluate the efficacy of Fungi-Free™ in the treatment of toe-nail fungal infection had significant improvement evidenced by softer, less symptomatic nails, suggesting efficacy of Fungi-Free™ [Data on file at Ganeden].

The above studies were preliminary in nature; therefore, *in vivo* study in animal model was needed to evaluate the efficacy of Fungi-Free™ in greater detail. In this study, we evaluated the efficacy of Fungi-Free™ cream in a guinea pig model of dermatophytosis. We used guinea pigs as they are susceptible to dermatophytosis similar to humans, and their large body surface provides sufficient area to perform experiments to determine clinical and mycological efficacy. Moreover, this model was used to evaluate potential antifungal agents that were subsequently approved for the treatment of dermatophytosis (such as, terbinafine). Therefore, a guinea pig dermatophytosis model that has been optimized and tested by our group was used to determine the efficacy of Fungi-Free™.

Body

Materials and Methods.

Laboratory Animals. The *in vivo* experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC), and the experimental procedures followed the Guidelines of the IACUC in compliance with the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Male albino guinea pigs (Harlan-Sprague-Dawley, San Diego, CA) with a body weight of 450-500 g were housed in Animal Resource Center assigned rooms under standard conditions. The animals were allowed to acclimate for a minimum of 5 days, and then they were randomly assigned to different experimental groups.

Fungal organism. *Trichophyton mentagrophytes* ATCC 24953, a pathogenic dermatophyte was selected as the infecting fungus. To prepare the challenge inoculum several petri dishes of potato dextrose agar (PDA; Difco laboratories, Detroit MI) were seeded with *T. mentagrophytes* and incubated at 30 °C for 5-7 days. The colonies were scraped from the plates in normal saline (NS) with sterile cell scrapers (Becton-Dickinson, MD). This cell suspension was centrifuged and washed twice in NS, then re-suspended to adjust cell count (1×10^8 conidia/ml) using a hemacytometer. The working solution was prepared fresh and used to inoculate the guinea pigs in these studies.

Animal inoculation. The inoculation procedure was done under anesthesia using intramuscular injection of anesthetic cocktail of acepromazine, ketamine and xylazine (1:3:3; v/v/v, 0.2 ml per animal). Using an electric shaver, an area on the left side of the guinea pig's back was clipped.

A closer shave of the clipped area was performed using a disposable razor. A 2.5 cm × 2.5 cm square outline was drawn on the guinea pig using a stencil and a marker pen. The marked area was abraded with sterile fine grit sandpaper. A cell suspension (1×10^7 *T. mentagrophytes* conidia in 100 µl of NS) was applied using a sterile pipette-tip and rubbed thoroughly on the abraded skin.

Antifungal agents. Fungi-FreeTM, bacterial supernatant, 2% miconazole and vehicle were provided by Ganeden Biotech Inc. (San Diego, CA).

Antifungal therapy. Topical medications were applied 72 hours post infection and continued through the next 7 days. A total of six groups of guinea pigs (10 per group) were included in this experiment: (i) infected untreated (negative) control, (ii) infected, treated with Fungi-FreeTM; (iii) infected, treated with vehicle containing bacterial supernatant only; (iv) infected, treated with vehicle only; (v) infected, treated with vehicle containing miconazole only (positive control) and (vi) uninfected untreated control. In this application, two studies were undertaken: study 1 was performed to compare the efficacies of the test and control agents administered once a day for a week, while study 2 was performed to compare the efficacies of the test and control agents administered twice a day for the same period.

Evaluation of Treatment Efficacy.

Clinical evaluation: The square infected area on the back of each guinea pig was divided into four quadrants. Clinical assessment of the stages of dermatophytosis in infected, treated and

untreated animals were scored on a scale from 0 to 5 as follows: 0, no signs of infection; 1, few slightly erythematous places on the skin; 2, well-defined redness, swelling with bristling hairs, bald patches, scaly areas; 3, large areas of marked redness, incrustation, scaling, bald patches, ulcerated in places; 4, partial damage to the integument, loss of hair; and 5, extensive damage to the integument and complete loss of hair at the site of infection. The scores were used to compare the efficacy of different treatment and control groups. Percent efficacy was calculated using the following equation:

$$\text{Percent Efficacy} = 100 - (T \times 100/C),$$

where T = total score of treatment arm and C = total score of untreated control. The total score for any group denotes the average clinical score from different animals in the same group.

Mycological evaluation: The hair root invasion test was used to assess mycological cure rate resulting from antifungal treatment (3, 4). Briefly, following clinical assessment, ten uprooted hairs per quadrant were planted on the surface of PDA. Plates were incubated at 30°C for 2 days. Following incubation, the numbers of hairs exhibiting fungal filaments at the hair root were counted. Percent efficacy was calculated using the following equation:

$$\text{Percent Efficacy} = 100 - (T \times 100/C),$$

where total score T = number of fungus-positive hairs in the treatment arm and C = number of fungus-positive hairs in the untreated control.

The total score for any group denotes the average number of fungus-positive hairs from different animals in the same group.

Histopathology Analysis: For histopathological examination, skin biopsy samples were obtained from one animal per group on day 13 of the study. With a disposable, sterile dermal biopsy punch (Miltex Instruments, Bethpage, New York), a piece of skin, 3-mm in diameter, was obtained from anesthetized animal representing the group. The tissue was fixed with 10% neutral buffered formalin (Evergreen Scientific, Los Angeles CA), embedded in paraffin and processed for histopathological examination. Fungal elements were visualized using Grocott Methenamine Silver (GMS) stain.

Statistical analyses. Student's t test was used to analyze the data obtained from two separate experiments performed on two different occasions. StatView software was used for all statistical analyses. A *P* value <0.05 was considered significant.

Key Research Accomplishments

Fungi-FreeTM cream applied topically once or twice a day for a week was found to be effective in the treatment of *T. mentagrophytes* -dermatophytosis in a guinea pig model. Bacterial supernatant, which is a component of Fungi- FreeTM cream, was also found to have anti-dermatophytic activity.

Reportable Outcomes

Fungi-FreeTM cream as well as miconazole (positive control) and bacterial supernatant (a component of Fungi-FreeTM) was tested for anti-dermatophytic activity. Study 1 and 2 were performed to evaluate the efficacies of the test agents administered once or twice a day for one week, respectively compared to controls.

Clinical efficacy.

Clinical efficacy study showed normal growth of hair and did not show any evidence of fungal infection in uninfected control guinea pigs. On the other hand, the infected, untreated control guinea pigs showed patches of hair loss and readily visible ulcerated or scaly skin. Figure 1A shows comparative clinical efficacy of each test compound administered once a day compared to infected untreated control or vehicle. Fungi-FreeTM was highly effective in the treatment of dermatophytosis compared to infected, untreated controls (94% efficacy; $P = <0.0001$). Similarly, miconazole, the positive control was also efficacious. The bacterial supernatant and vehicle also showed some activity compared to the negative control ($P = <0.05$, for both comparisons).

The relative clinical efficacy of Fungi-FreeTM and controls administered twice daily are shown in Figure 1B. As can be seen, Fungi-FreeTM demonstrated high clinical efficacy compared to infected untreated control ($P < 0.0002$). As expected, the positive control also led to clinical cure ($P = 0.0001$). Animals treated with bacterial supernatant showed clinical improvement, which was superior to the vehicle alone ($P = 0.012$). As shown in Table 1, a 2.5-fold improvement in the efficacy of bacterial supernatant was noted with twice daily treatment compared to once daily treatment.

Mycological efficacy.

Figure 2A shows comparative mycological efficacy of each test compound administered once a day. Mycological study revealed that Fungi-FreeTM was highly effective in inhibiting fungal growth compared to infected, untreated control (Efficacy 99.3%; $P < 0.0001$). Similar efficacy was noted for the positive control (see Table 1). Guinea pigs treated with bacterial supernatant also showed mycological activity ($P < 0.05$). Although the vehicle showed some inhibitory activity relative to the negative control, the mycological efficacy of bacterial supernatant was significantly greater than the that of the vehicle ($P < 0.04$).

Figure 2B shows comparative mycological efficacy of Fungi-FreeTM and controls administered twice daily. Mycological study revealed that Fungi-FreeTM was highly effective in inhibiting fungal growth compared to infected, untreated control (efficacy 100%; $P = 0.0001$). Similar mycological efficacy was noted for the positive control (see Table 1). A modest inhibitory activity was observed in animals treated with bacterial supernatant or vehicle.

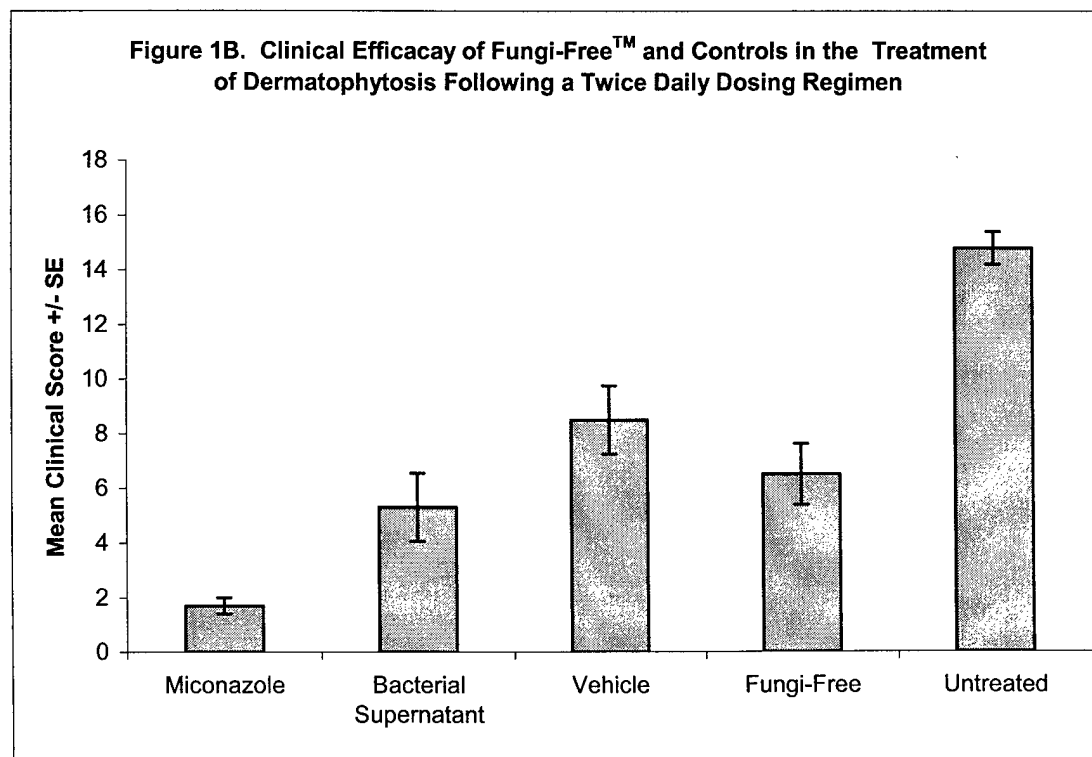
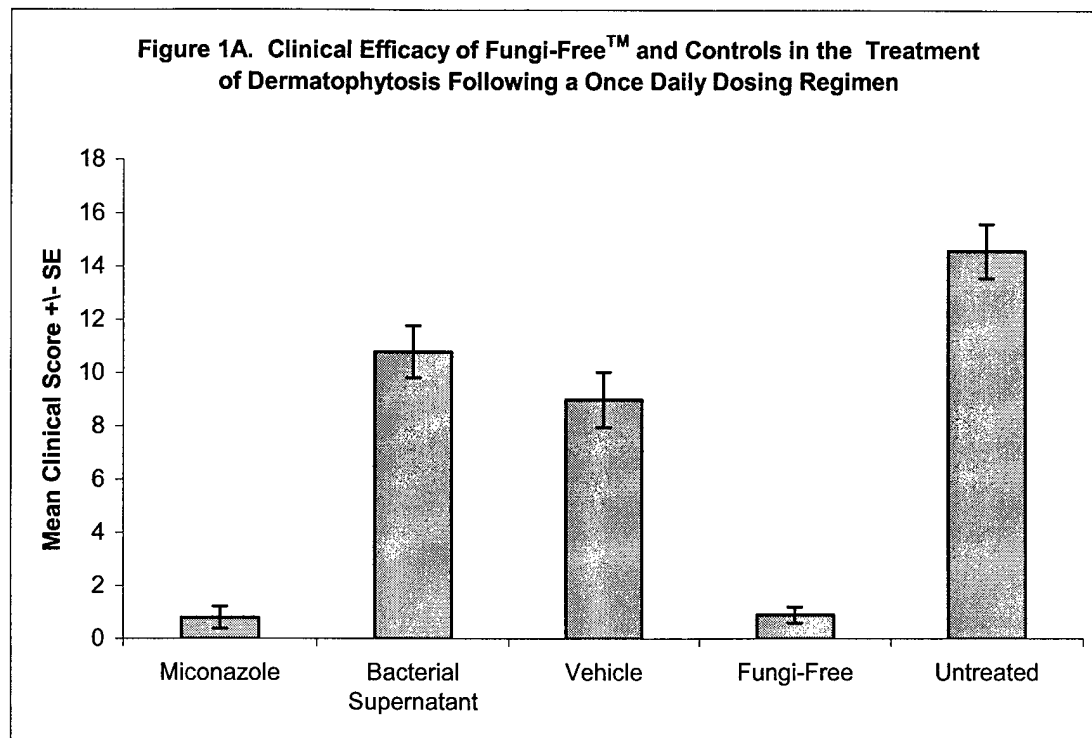


Figure 2A. Mycological Efficacy of Fungi-Free™ and Controls in the Treatment of Dermatophytosis Following a Once Daily Dosing Regimen

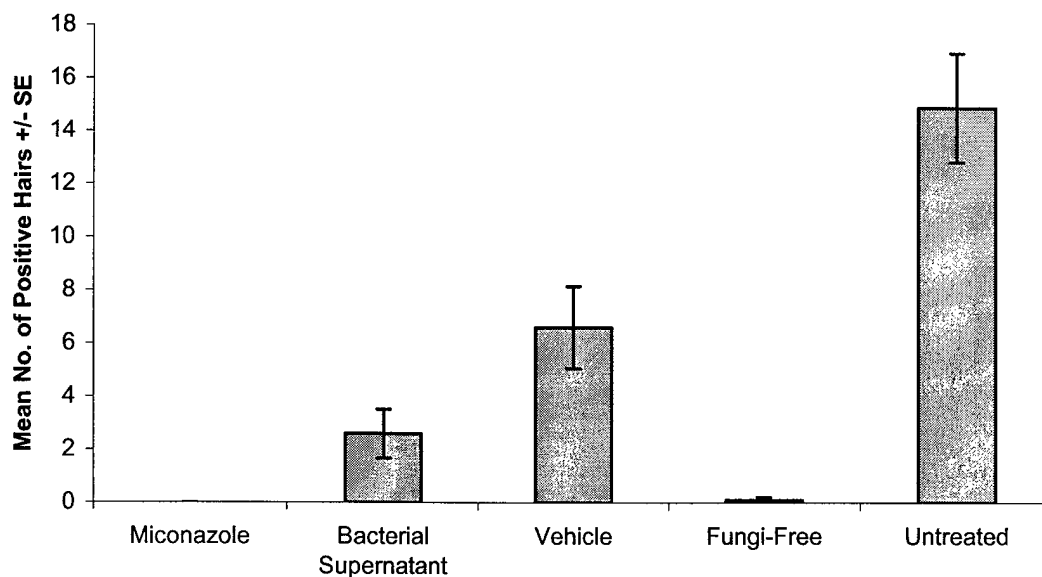


Figure 2B. Mycological Efficacy of Fungi-Free™ and Controls in the Treatment of Dermatophytosis Following a Once Daily Dosing Regimen

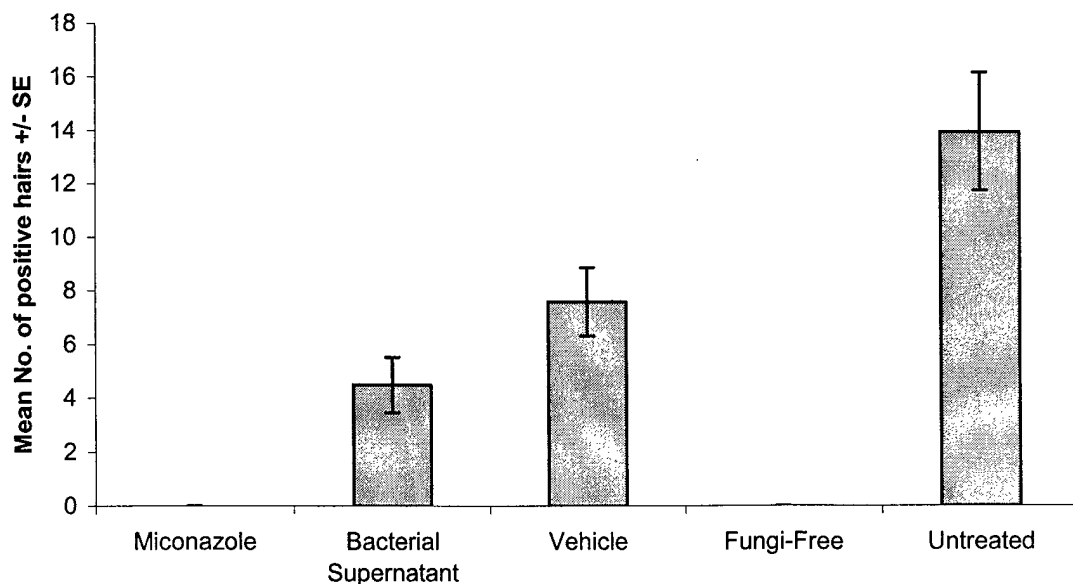


TABLE 1. Summary of Clinical and Mycological Efficacy of Different Treatment Groups.

| Treatment Group | Clinical Efficacy | | Mycological Efficacy | |
|---------------------------------|----------------------|-----------------------|----------------------|-----------------------|
| | once daily treatment | twice daily treatment | once daily treatment | twice daily treatment |
| Fungi-Free | 93.84% | 56.08% | 99.33% | 100% |
| Miconazole + Vehicle | 94.52% | 88.51% | 100% | 100% |
| Bacterial Supernatant + Vehicle | 26.03% | 64.19% | 82.55% | 67.86% |
| Vehicle | 38.36% | 42.57% | 55.7% | 45.71% |

Histopathology.

In addition to evaluation of clinical and mycological efficacy, histopathology was performed on one randomly selected animal from each group. As expected, no fungal elements, and normal histological features were observed in GMS-stained processed skin sections obtained from uninfected animal. Fungal elements were detectable in skin sections from guinea pig infected with *T. mentagrophytes*, indicating successful infection (Data not shown). No fungal elements or any evidence of tissue destruction were detected in skin sections obtained from animals treated with Fungi-FreeTM. Also no fungal elements were detected in skin from animals treated with miconazole, bacterial supernatant or vehicle.

Conclusion

Fungi-FreeTM is an effective therapy for the treatment of dermatophytosis and provides clinical and mycological cure in guinea pig. The bacterial supernatant, which is a component of Fungi-FreeTM also exhibited antifungal efficacy. These studies indicate that Fungi-FreeTM may have utility in the treatment of dermatophytosis.

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